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Food quality of heterotrophic bacteria for *Daphnia magna*: evidence for a limitation by sterols

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Abstract

The quality of heterotrophic bacteria as food for metazoan grazers has been investigated poorly. We conducted growth experiments with juvenile *Daphnia magna* feeding on different strains of heterotrophic bacteria that represent typical pelagic bacteria of five phylogenetically distinct groups. The bacterial food suspensions were supplemented with cholesterol and/or the polyunsaturated fatty acid eicosapentaenoic acid (EPA), two essential nutrients that are either absent or scarcely represented in bacteria. Our data imply that the selected heterotrophic bacteria are of poor food quality for *D. magna*, which was indicated either by very low somatic growth rates or by high mortality. However, with four out of six bacterial strains tested, the somatic growth rates increased significantly upon supplementation with cholesterol, which shows that the lack of sterols in bacteria is a major food quality constraint. We did not find clear evidence for a limitation by EPA on bacterial diets within our growth experiments. High mortality was observed when *D. magna* was fed with *Hydrogenophaga* sp. or *Pseudomonas* sp., which indicates that these two bacterial strains are toxic to *D. magna*. Our findings highlight the limitations of bacteria as a carbon source for *Daphnia* and point to a so far underestimated diversity of interactions between grazers and its bacterial food.

Introduction

Heterotrophic bacteria constitute a substantial part of the suspended particulate organic matter in many aquatic ecosystems, thereby forming the base of a complex microbial food web (Azam *et al.*, 1983; Sherr & Sherr, 1984). Hetero trophic flagellates and ciliates prey upon bacteria and are able to restructure bacterial communities towards grazing resistant forms and to suppress bacterial biomass produc tion (Sherr & Sherr, 1984; Fenchel, 1986; Jürgens *et al.*, 1994). Top down effects of metazoan grazers on hetero trophic bacteria are mediated either indirectly by trophic cascades, i.e. by suppression of bacterivorous protozoans, or by direct consumption of bacteria (Langenheder & Jürgens, 2001; Degans *et al.*, 2002). In particular, filter feeding cladocerans of the genus *Daphnia*, the key herbivores in many freshwater ecosystems, efficiently consume hetero trophic bacteria (e.g. Gophen & Geller, 1984; Brendelberger, 1991; Pace & Cole, 1994) and, like bacterivorous protozoans, are able to shape the bacterial community structure and to suppress bacterial biomass production (Jürgens, 1994; Lan genheder & Jürgens, 2001; Degans *et al.*, 2002).

Daphnia are largely nonselective filter feeders, which do not discriminate between food particles with regard to their nutritional quality (DeMott, 1986). Analysis of stable isotope patterns and fatty acid biomarkers revealed that heterotrophic bacteria can contribute significantly to the nutrition of *Daphnia* species (Perga *et al.*, 2006; Taipale *et al.*, 2008, 2009). However, the quality of heterotrophic bacteria as food for *Daphnia* has not been investigated thoroughly. Compared with many algae, bacteria are super ior competitors for phosphorus and often characterised by high P:C values (Vadstein, 2000), suggesting that bacteria are a rich source of phosphorus (Hessen & Andersen, 1990). Besides phosphorus, however, daphnids rely on a dietary source of essential biochemicals, i.e. polyunsaturated fatty acids (PUFAs) (Wacker & von Elert, 2001; von Elert, 2002) and sterols (Martin Creuzburg *et al.*, 2005b, 2008, 2009),

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which are either absent or hardly represented in bacteria (Napolitano, 1998; Russell & Nichols, 1999; Volkman, 2003). PUFAs and sterols are indispensable structural components of cell membranes, and both serve as precursors for a number of bioactive molecules. For instance, the long chain PUFAs arachidonic acid and eicosapentaenoic acid (EPA) serve as precursors for prostaglandins, which are thought to be relevant in arthropod reproduction (Harrison, 1990). Sterols, on the other hand, serve as precursors for steroid hormones, such as ecdysteroids, which are involved in the process of molting (Grieneisen, 1994; Martin Creuzburg *et al.*, 2007). Laboratory experiments revealed that daphnids feeding on a cyanobacterial diet are simultaneously limited by the absence of sterols and long chain PUFAs (Martin Creuzburg *et al.*, 2008, 2009). Here, we investigated the role of sterols and long chain PUFAs in determining the quality of selected heterotrophic bacteria as food for *Daphnia*. We hypothesized that the absence of essential lipids is a general food quality constraint of prokaryotic food sources. In standardized growth experiments, juvenile *Daphnia magna* were fed with different strains of heterotrophic bacteria that represent typical pelagic bacteria from five phylogenetic groups (Glöckner *et al.*, 2000; Pearce *et al.*, 2005; Van der Gucht *et al.*, 2005). The bacterial food suspensions were supplemented with sterol and/or EPA containing liposomes to assess the significance of a dietary deficiency in these lipids in determining the food quality of these heterotrophic bacteria.

Materials and methods

Cultivation of food organisms and preparation of food suspensions

Growth experiments were conducted with five strains of heterotrophic bacteria representing typical freshwater microorganisms from different taxonomic groups (*Pseudomonas* sp. DD1, *Microbacterium* sp. DD4, *Flavobacterium* sp. DD5b, *Hydrogenophaga* sp. DD8b, *Caulobacter* sp. BSL1) and one novel as yet undescribed methanotrophic bacterium (Table 1). The strains were isolated from the guts of *D. magna* or from water/sediment samples taken from Lake Constance, respectively. They were identified via nearly full length 16S rRNA gene sequencing by GATC (Konstanz, Germany) after PCR amplification (cf. Freese *et al.*, 2010) and purification (Zymo DNA Clean and Concentrator). The sequences were visually controlled, aligned using BIOEDIT software, version 7.0.5.3 (Hall, 1999), and finally compared with sequences in the GenBank database using the BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST) (Altschul *et al.*, 1997). The sequence data were submitted to GenBank and were provided with GenBank accession numbers (Table 1).

The bacterial strains were grown in mineral medium containing 0.03 M Na K phosphate buffer, pH 7.2 (1 L), NH_4Cl ($1\,g\,L^{-1}$), $MgSO_4 \cdot 12H_2O$ ($0.5\,g\,L^{-1}$), $CaCl_2 \cdot 6H_2O$ ($5\,mg\,L^{-1}$), $10\,mL\,L^{-1}$ vitamin solution (cyanocobalamin $50\,mg\,L^{-1}$, 4 aminobenzoic acid $50\,mg\,L^{-1}$, biotin $10\,mg\,L^{-1}$,

Table 1. Characterization of bacterial food strains [phylogenetic affiliation and NCBI GenBank accession number, isolation source, carbon source (YE, yeast extract), and end concentration of bacterial carbon and abundance in experiments 1 (E1) and 2 (E2)]

		Nearest related published					
Bacterial strain	Phylogenetic group (NCBI accession #)	Strain	Similarity (%)	Isolation source	Carbon source for cultivation	Bacterial C (mg C L^{-1})	Abundance (mL^{-1})
Pseudomonas sp. DD1	*Gammaproteobacteria* (HQ113379)	*Pseudomonas gessardii* CIP 105469 (NR 024928)	99.9	*Daphnia*, digestive tract	E1: 20 mM glucose E2: 20 mM glucose+0.1% YE	E1: 0.28 E2: 4.34	E1: 6.6×10^6 E2: 4×10^7
Microbacterium sp. DD4	*Actinobacteria* (HQ113380)	*Microbacterium kitamiense* strain kitami C2 (AB013907)	100	*Daphnia*, digestive tract	E1: 20 mM glucose+0.1% YE E2: 20 mM glucose+0.1% YE	E1: 0.37 E2: 2.45	E1: 6.6×10^6 E2: 4×10^7
Flavobacterium sp. DD5b	*Bacteroidetes; Flavobacteria* (HQ113381)	*Flavobacterium* sp. MH45 (EU182873)	98.8	*Daphnia*, digestive tract	E1: 20 mM glucose+0.1% YE E2: 20 mM glucose	E1: 0.33 E2: 3.61	E1: 6.6×10^6 E2: 4×10^7
Hydrogenophaga sp. DD8b	*Betaproteobacteria* (HQ113382)	*Hydrogenophaga pseudoflava* (NR 028717)	99.5	*Daphnia*, digestive tract	E1: M1 (peptone+yeast extract) E2:	E1: 0.42 E2:	E1: 6.6×10^6 E2:
Caulobacter sp. BSL1	*Alphaproteobacteria* (HQ113383)	*Caulobacter henricii* ATCC 15253 (NR 025319)	99.6	Lake Constance littoral water	E1: E2: 20 mM fructose+glucose	E1: E2: 2.25	E1: E2: 4×10^7
Methanotrophic bacterium	*Gammaproteobacteria*			Lake Constance sediment	E1: E2: 30% methane	E1: E2: 2.55	E1: E2: 1.3×10^7

pantothenate 25 mg L^{-1}, nicotinic acid 100 mg L^{-1}, pyridoxamine 250 mg L^{-1}, thiamine 50 mg L^{-1}) (Widdel & Pfennig, 1981), 1 mL L^{-1} trace element solution (SL10, Atlas, 1997) (10 mL L^{-1}) of either glucose, glucose and fructose, or glucose enriched with yeast extract as carbon sources to increase the growth rates and biomass production (Table 1). *Hydrogenophaga* sp. was cultivated in M1 (nutrient broth) to obtain high biomass, and the methanotroph was grown under 30% methane/70% air. Bacteria were grown at 20 °C and harvested daily in the late exponential and early stationary phases. Cells were centrifuged (10 min, 4500 ***g***) washed, and resuspended in sterile filtered and autoclaved Lake Constance water. Aggregates were dissolved by vortexing and sonication for 30 s. In order to add defined numbers of bacteria to the growth experiments (Table 1), cell numbers were determined in a Helber counting chamber using a Zeiss Axiophot microscope.

The green alga *Scenedesmus obliquus* (SAG 276 3a) was used as food for stock cultures of *D. magna* and as a reference food in the growth experiments. It was grown in semi continuous batch cultures as described in Martin Creuzburg *et al.* (2005b) and harvested in the late exponential growth phase. Carbon contents of the autotrophic food suspensions were estimated from photometric light extinctions (800 nm) and from previously determined carbon extinction equations.

Daphnia growth experiments

Stock cultures of *D. magna* (originally isolated by Lampert, 1991) were raised in filtered lake water (0.2 µm pore sized membrane filter) and saturating concentrations of *S. obliquus*. Growth experiments were carried out with third clutch neonates (born ± 6 h) at 20 °C in glass beakers filled with 200 mL of filtered lake water (< 0.2 µm). Each treatment consisted of three replicates with five *D. magna* per beaker. Animals were transferred daily into new beakers with freshly prepared food suspensions. Two experiments were conducted with different food concentrations. In the first experiment, daphnids were exposed to low food concentrations, i.e. 6.6×10^{6} cells mL^{-1}, which is equivalent to approximately 0.3 0.4 mg C L^{-1}. In the second experiment, bacteria were provided at high concentrations, i.e. 4 (in one case 1.3) $\times 10^{7}$ cells mL^{-1}, equivalent to approximately 2 2 4.3 mg C L^{-1} (Table 1).

In both experiments, bacterial food suspensions were supplemented with either 100 µL of control liposomes (no sterols, no PUFAs), 50 µL cholesterol containing liposomes, 50 µL EPA containing liposomes, or simultaneously with 50 µL cholesterol and 50 µL EPA containing liposomes per beaker. The two treatments that contained either cholesterol or EPA containing liposomes were additionally supplemented with 50 µL of control liposomes to maintain constant carbon concentrations, i.e. each beaker was supplemented in total with 100 µL of liposome suspension. Liposome stock suspensions were prepared as described in Martin Creuzburg *et al.* (2008). Both experiments were completed by a concomitant starvation treatment and a treatment containing 2 mg C L^{-1} of *S. obliquus*. Subsamples of the experimental animals were taken at the beginning and at the end of an experiment, dried for 24 h, and weighed on an electronic balance (Mettler Toledo XP2U; ± 0.1 µg). Juvenile somatic growth rates (g) were determined as the increase in dry mass from day 0 (M_0) to day 6 (M_6) of the experimental period ($t = 6$ days) using the equation: $g = (\ln M_6 - \ln M_0)/t$. Clutch sizes were estimated by counting the eggs in the brood chambers of the animals at the end of each experiment.

Fatty acid and sterol analyses of bacterial food suspensions

For analysis of fatty acids and sterols, at least 5×10^{9} cells were harvested by centrifugation, washed, freeze dried, and stored at − 80 °C. Total lipids were extracted three times from freeze dried samples with dichloromethane/methanol (2 : 1 v/v) and the pooled cell free extracts were evaporated to dryness with nitrogen. Lipid extracts were transesterified with 3 mol L^{-1} methanolic HCl (60 °C, 15 min) for analysis of fatty acids or saponified with 0.2 mol L^{-1} methanolic KOH (70 °C, 1 h) for analysis of sterols. Subsequently, fatty acid methyl esters (FAMEs) were extracted three times with 2 mL iso hexane; the neutral lipids were partitioned into iso hexane : diethyl ether (9 : 1 v/v). The lipid containing fraction was evaporated to dryness under nitrogen and resuspended in a volume of 10 20 µL iso hexane. Lipids were analyzed by GC on an HP 6890 GC equipped with a flame ionization detector (FID) and a DB 225 (J&W Scientific) capillary column to analyze FAMEs or with an HP 5 (Agilent) capillary column to analyze sterols. The details of the GC configurations are given elsewhere (for fatty acids, Martin Creuzburg *et al.*, 2010; for sterols, Martin Creuzburg *et al.*, 2009). Lipids were quantified by comparison with internal standards (C23:0 ME; 5α cholestan) using FID and identified by their retention times (bacterial acid methyl ester mix, Sigma Aldrich) and their mass spectra, which were recorded using a gas chromatograph mass spectrometer (Finnigan MAT GCQ) equipped with a fused silica capillary column (DB 225MS, J&W Scientific for FAMEs; DB 5MS, Agilent for sterols). Sterol samples were analyzed in their free form and as their trimethylsilyl derivatives. Spectra were recorded between 50 and 600 amu in the EI ionization mode. The limit of quantitation was approximately 20 ng for fatty acids or sterols. The absolute amount of each lipid was related to the particulate organic carbon (POC), which was determined from bacterial suspensions concentrated in tin capsules for liquid samples using an NCS 2500 analyzer (ThermoQuest GmbH, Egelsbach, Germany).

Statistical analysis

Somatic growth rates of *D. magna* were analyzed using one way ANOVA. Treatments in which only one or no animal per beaker survived were excluded from the ANOVAS. ANOVAS were carried out using the general linear model module of STATISTICA 6.0 (StatSoft Inc., Tulsa, OK). Raw data met the assumption of homogeneity of variance; treatment effects were tested using Tukey's honestly significant difference (HSD) *post hoc* tests.

Results

Sterol and fatty acid composition of the food sources

Sterols could not be detected in any of the bacterial strains. Chondrillasterol [(22E) 5α poriferasta 7,22 dien 3β ol], fungisterol (5α ergost 7 en 3β ol), and 22 dihydrochondrillasterol (5α poriferast 7 en 3β ol) were the principal sterols found in the green alga *S. obliquus* (in total 7.1 ± 0.7 $\mu g\,mg^{-1}$ C). The fatty acid composition of all the bacterial strains was dominated by short chain saturated (14:0, 16:0, 18:0) and monounsaturated fatty acids (16:1n 7, 17:1n 7, 18:1n 9/n 12). Significant amounts of branched chain fatty acids (iso and/or anteiso 15:0) were detected in *Caulobacter* sp., *Microbacterium* sp., and *Flavobacterium* sp. PUFAs could not be detected in any of the bacterial strains. The green alga *S. obliquus* contained high amounts of 18:2n 6 and 18:3n 3, but no PUFAs with more than 18 carbon atoms.

The supplemented liposomes did not differ in their contents of palmitic acid (16:0) and oleic acid (18:1n 9), which are both components of the phospholipids used to prepare the liposomes (Martin Creuzburg *et al.*, 2008). Liposomes prepared in the presence of EPA contained considerable amounts of this fatty acid (12.8 μg in 50 μL of liposome stock suspension) and liposomes prepared in the presence of cholesterol contained considerable amounts of this sterol (14.1 μg in 50 μL of liposome stock suspension), but neither EPA nor cholesterol was detected in liposomes prepared without supplementing these compounds.

Somatic growth rates, clutch sizes, and mortality of *D. magna*

In general, juvenile somatic growth rates of *D. magna* grown on unsupplemented bacteria were much lower than somatic growth rates obtained with the green alga *S. obliquus* (Figs 1 and 2). In the first experiment with low food concentrations (Fig. 1), the growth rates of *D. magna* were significantly reduced when *Microbacterium* sp. and *Flavobacterium* sp. were supplemented with control liposomes or EPA containing liposomes (Tukey's HSD, $P < 0.05$ following ANOVA: *Microbacterium* sp. $F_{4,10} = 72.05$; *Flavobacterium* sp. $F_{4,10} = 214.56$, both $P < 0.001$). In contrast, the growth rates of *D. magna* fed either *Microbacterium* sp. or *Flavobacterium* sp. were significantly improved by cholesterol supplementation (Tukey's HSD, $P < 0.05$). Simultaneous supplementation with cholesterol and EPA further improved the somatic growth rates of daphnids fed *Flavobacterium* sp., but not of daphnids fed *Microbacterium* sp. Somatic growth rates of daphnids fed *Pseudomonas* sp. were all negative and not affected by supplementation (ANOVA, $F_{4,10} = 1.22$, $P = 0.36$). *Daphnia magna* did not produce eggs within the experimental period (6 days) in any of the bacterial treatments, except for a single animal, which was fed with cholesterol and EPA supplemented *Flavobacterium* sp. *Daphnia magna* fed the green alga *S. obliquus* produced 9.1 ± 1.4 eggs per individual (± SD) within the experimental period. In the starvation treatment, all animals died within 4 days. Animals fed *Hydrogenophaga* sp. all died within 2 days of feeding, irrespective of lipid supplementation (Fig. 3). In all the other food treatments, mortality was negligible.

In the second experiment with high food concentrations (Fig. 2), supplementation of bacterial food suspensions with control liposomes or EPA containing liposomes did not affect the somatic growth rates of *D. magna* (Tukey's HSD, $P > 0.05$ following ANOVA: *Microbacterium* sp., $F_{4,10} = 76.26$; *Caulobacter* sp., $F_{4,10} = 82.48$; methanotrophic bacterium, $F_{4,10} = 39.57$; all $P < 0.001$). The growth rates of animals fed *Microbacterium* sp., *Caulobacter* sp., or the methanotrophic bacterium were all significantly improved by cholesterol supplementation (Tukey's HSD, $P < 0.05$). Without cholesterol supplementation, animals fed *Flavobacterium* sp. did not survive the experimental period and died even faster than starved daphnids (Fig. 3). The growth rates of animals fed *Flavobacterium* sp. or *Caulobacter* sp. increased upon cholesterol supplementation almost to the level obtained with the green alga *S. obliquus* (Fig. 2). Simultaneous supplementation of the bacterial food suspensions with cholesterol and EPA containing liposomes did not further improve the somatic growth rates of *D. magna* (Tukey's HSD, $P > 0.05$; ANOVA for *Flavobacterium* sp., $F_{1,4} = 0.002$, $P = 0.96$). The growth rates of *D. magna* fed *Pseudomonas* sp. were not calculated, because only single animals survived the experimental period (Fig. 3). The animals did not die as quickly as when exposed to *Hydrogenophaga* sp. (first experiment), but their number decreased continuously after the third day of incubation even more rapidly than the starved animals. Mortality was slightly reduced when *Pseudomonas* sp. was supplemented with control liposomes or EPA containing liposomes, but these animals hardly survived the experimental period. In all the other bacterial food treatments, mortality was negligible. *Daphnia magna* did not produce eggs in any of the bacterial food treatments, except for animals fed cholesterol supplemented *Flavobacterium* sp. (+cholesterol: 7.8 ± 0.9 eggs per individual;+cholesterol+EPA: 0.7 ± 0.6 eggs individual). Animals fed the green alga *S. obliquus* produced 9.4 ± 1.4 eggs per individual.

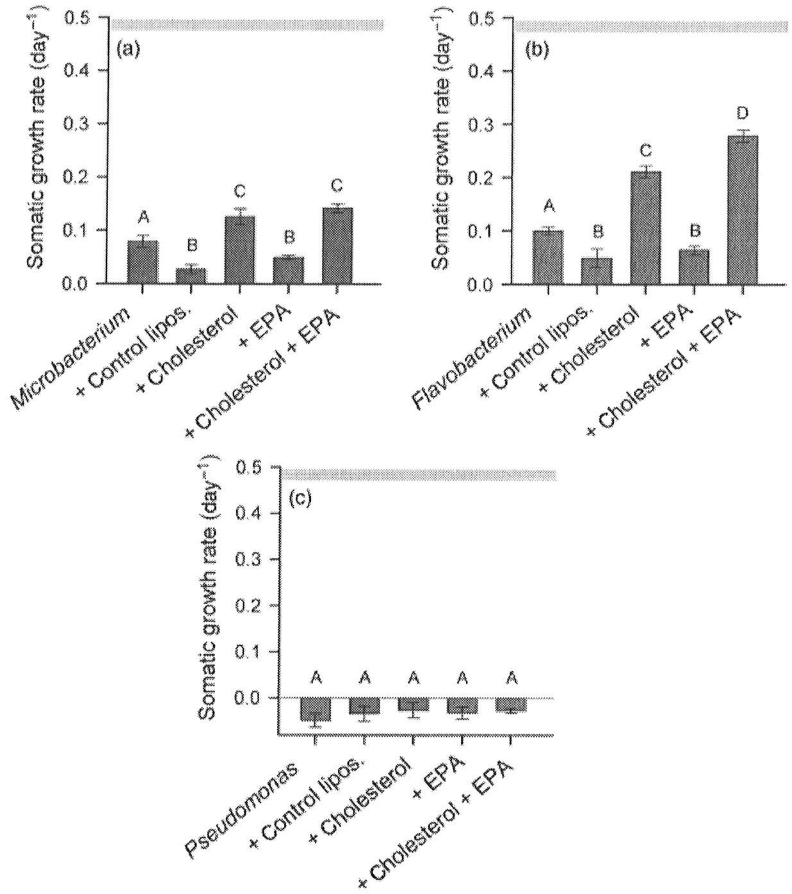

Fig. 1. Juvenile somatic growth rates of *Daphnia magna* exposed to low concentrations of (a) *Microbacterium* sp., (b) *Flavobacterium* sp., or (c) *Pseudomonas* sp. either unsupplemented or supplemented with EPA and/or cholesterol containing liposomes. Control liposomes were prepared without adding EPA or cholesterol. The horizontal gray bar indicates the somatic growth rates of *D. magna* fed the green alga *Scenedesmus obliquus*, which was used as a reference food. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$ following ANOVA).

Discussion

Essential lipids and food quality of bacteria

Here, we have shown that the heterotrophic bacteria used in our study were of poor food quality for *D. magna* when they were provided as the sole food source, which was indicated by very low somatic growth rates or increased mortality. With four out of six bacterial strains tested, the somatic growth rates of *D. magna* increased significantly upon sterol supplementation. Hence, the absence of sterols in bacteria is a major food quality constraint. The positive effect of sterol supplementation was in general more pronounced at high bacterial food supply (second experiment), which adds to the general view that the effects of food quality increase with food quantity (Sterner, 1997). The most prominent effects were observed when *D. magna* was fed high concentrations of *Flavobacterium* sp. (*Bacteroidetes*) and *Caulobacter* sp. (*Alphaproteobacteria*), which both represent ubiquitous bacteria common in aquatic habitats (e.g. Pearce *et al.*, 2005; Lapteva *et al.*, 2007). In both cases, sterol supplementation led to high somatic growth rates, which increased almost to the level obtained with the green alga *S. obliquus.* Sterol supplementation also improved the food quality of *Microbacterium* sp. (*Actinobacteria*) and of the methanotrophic bacterium (*Gammaproteobacteria*) and, hence, of bacteria belonging to phylogenetically highly distinct groups. In accordance with previous studies, this indicates that a dietary source of sterols is required for the somatic growth of *Daphnia* species (Martin Creuzburg *et al.*, 2005b, 2008, 2009). Without sterol supplementation, animals exposed to high concentrations of *Flavobacterium* sp. did not survive the experimental period and died even faster than starved animals, which suggests that *Flavobacterium* sp. provided as the sole food source in high concentrations is harmful for *D. magna* and that this putatively adverse effect disappears in the presence of cholesterol. However, the mechanisms underlying this observation remain unclear and should be subjected to further investigations.

Irrespective of the bacterial strain used as food, supplementation with EPA did not improve the somatic growth rates of *D. magna*, which suggests that somatic growth on a bacterial diet is not affected by a dietary EPA deficiency. The

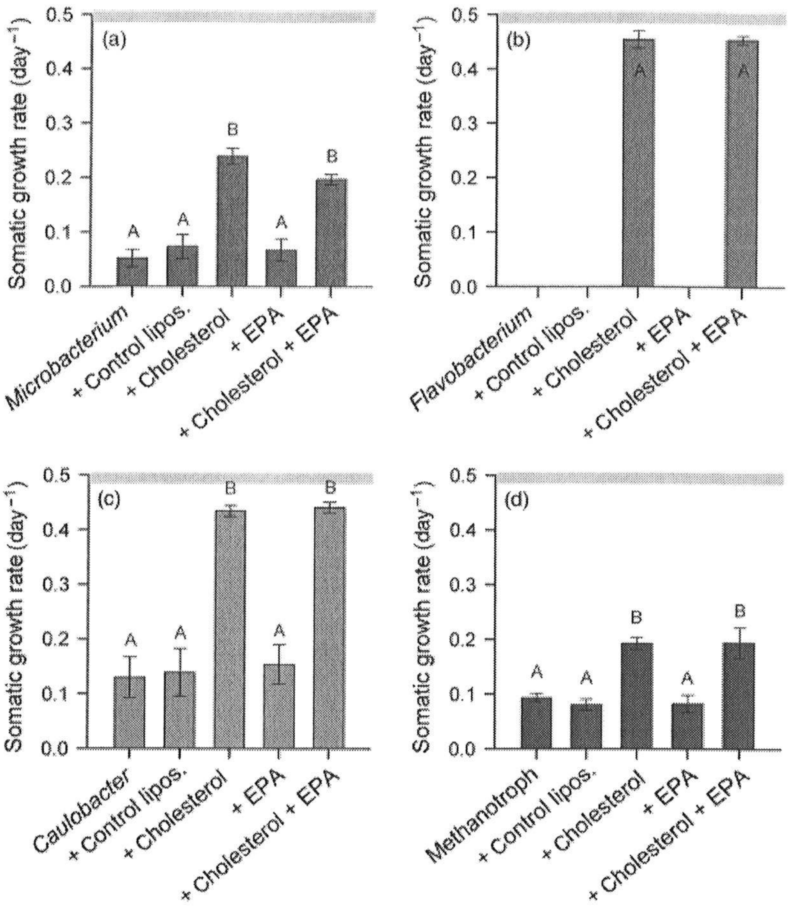

Fig. 2. Juvenile somatic growth rates of *Daphnia magna* exposed to high concentrations of (a) *Microbacterium* sp., (b) *Flavobacterium* sp., (c) *Caulobacter* sp., or (d) a methanotrophic bacterium either unsupplemented or supplemented with EPA and/or cholesterol containing liposomes. Control liposomes were prepared without adding EPA or cholesterol. The horizontal gray bar indicates the somatic growth rates of *D. magna* fed the green alga *Scenedesmus obliquus*, which was used as a reference food. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$ following ANOVA).

only positive effect on EPA supplementation was observed in the first experiment with *Flavobacterium* sp. as food. In this case, simultaneous supplementation with cholesterol and EPA led to slightly increased somatic growth rates compared with cholesterol supplementation alone, a pattern that had been observed previously with cyanobacterial food suspensions (Martin Creuzburg *et al.*, 2008, 2009). Interestingly, this positive effect on EPA supplementation disappeared when *Flavobacterium* sp. was provided at high concentrations (second experiment). In previous studies, we have shown that dietary EPA is primarily important for reproduction of *D. magna* (Martin Creuzburg *et al.*, 2008, 2009). In the first experiment, *D. magna* did not produce eggs within the experimental period in any of the bacterial treatments, except for a single animal that was fed *Flavobacterium* sp. supplemented with cholesterol and EPA simultaneously. In the second experiment, *D. magna* produced eggs only when fed cholesterol supplemented *Flavobacterium* sp., but additional EPA supplementation reduced egg production. Overall, we did not find clear evidence for a limitation by EPA on bacterial diets within our 6 day long growth experiments. It remains to be tested, however, whether the effects of EPA supplementation of a bacterial diet become clearer when more than one reproduction cycle is considered (cf. Martin Creuzburg *et al.*, 2009).

Toxicity of bacteria

It has been recognized that a number of bacterial strains isolated from aquatic habitats produce a variety of secondary metabolites that are biologically active towards protozoan and metazoan grazers and hence are discussed as antipredator compounds (Jensen & Fenical, 1994; Matz & Kjelleberg, 2005; Matz *et al.*, 2008). For instance, the purple pigment violacein, which was extracted from three different bacterial genera (*Chromobacterium*, *Janthinobacterium*, and *Pseudoalteromonas*), has been shown to act as a potent toxin against several freshwater bacterivores, among them *D. magna* (Deines *et al.*, 2009). In our study, juvenile *D. magna* feeding on *Hydrogenophaga* sp. died rapidly irrespective of lipid supplementation, indicating high toxicity. This is an interesting finding especially because this *Hydrogenophaga*

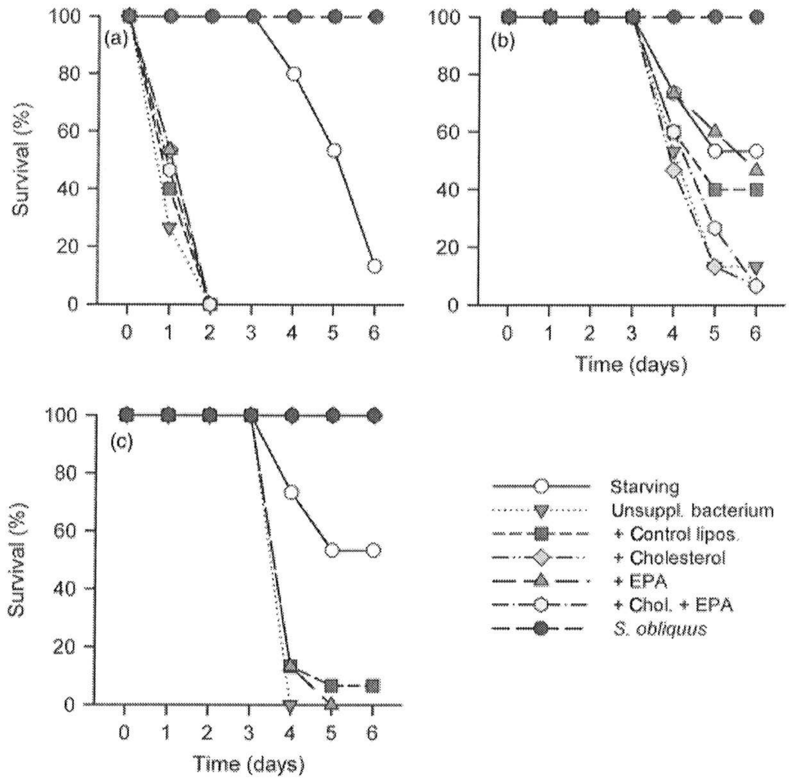

Fig. 3. Survival of juvenile *Daphnia magna* exposed to (a) low concentrations of *Hydrogenophaga* sp. or high concentrations of (b) *Pseudomonas* sp. and (c) *Flavobacterium* sp. either unsupplemented or supplemented with EPA- and/or cholesterol-containing liposomes. Data were calculated from the numbers of individuals that survived the experimental period of 6 days (means of $n = 3$ jars). The survival of starved animals and of animals fed the green alga *Scenedesmus obliquus* is shown for comparison.

strain was isolated from the gut of adult *D. magna* and belongs to the same family (*Comamonadaceae*) as *Limnohabitans* sp., which dominates the bacterial community within the gut of *D. magna* (Qi *et al.*, 2009; H.M. Freese & B. Schink, pers. commun.).

High mortality was also observed when animals were exposed to high concentrations of a *Pseudomonas* strain that was also isolated from the guts of adult *D. magna*. Species of the genus *Pseudomonas* belong to the most common bacteria in aquatic habitats (Pearce *et al.*, 2005) and previously have been found to be associated with *Daphnia* (Qi *et al.*, 2009). Some *Pseudomonas* species/strains are known as vertebrate and invertebrate pathogens, such as *Pseudomonas aeruginosa* (Ziprin & Hartman, 1971; Tan *et al.*, 1999; Jander *et al.*, 2000), or the recently described *Pseudomonas entomophila*, which can orally infect and kill *Drosophila* and other insect species (Vodovar *et al.*, 2005). *Pseudomonas* strains have been shown to produce secondary metabolites that inhibit or kill various invertebrates, including *Daphnia* (Sinden *et al.*, 1971; Padmanabhan *et al.*, 2005), or to slowly kill their hosts via an infection-like process while accumulating in their intestine after consumption (Tan *et al.*, 1999). The differences in the strength of the negative effects observed in the two experiments in our study might be due to the different concentrations at which *Pseudomonas* sp. was fed to *D. magna* and hence to the different concentrations of toxic secondary metabolites potentially produced by this *Pseudomonas* strain. It is also possible that only at lower dietary *Pseudomonas* sp. concentrations an already established intestinal microbiota was able to prevent colonization and infection by these bacteria. Further studies are needed to reveal the significance of different *Hydrogenophaga* and *Pseudomonas* strains to act as pathogens in the digestive tracts of *Daphnia* species and to identify the toxic compounds potentially produced by these bacterial strains to be able to assess the relevance of a *Hydrogenophaga* or *Pseudomonas* virulence in the field.

Ecological implications

Our data support the hypothesis that a high share of prokaryotic food sources within the edible size fraction of Lake Seston leads to a sterol limitation of *Daphnia* species, as prokaryotes generally do not contain sterols (Volkman, 2003; Summons *et al.*, 2006). This might be important in eutrophic lakes, in particular during cyanobacterial blooms in which cyanobacteria dominate the phytoplankton (Martin-Creuzburg *et al.*, 2008), but also in meso- to oligotrophic clearwater and humic lakes in which heterotrophic bacteria can account for the major fraction of suspended POC (Hessen, 1985; Simon *et al.*, 1992). In previous experiments, using different mixtures of the sterol-containing green alga

S. obliquus and the sterol free picocyanobacterium *Synechococcus elongatus* as food, we have estimated that *Daphnia* species require at least 50% of the green alga, i.e. eukaryotic carbon, in their diet to compensate for a dietary sterol deficiency (Martin Creuzburg *et al.*, 2005b). Hence, the ratio between pro and eukaryotic carbon within Lake Seston may determine whether or not *Daphnia* species are limited by a low availability of sterols, which in turn may affect the efficiency with which prokaryotic carbon is transferred to higher trophic levels.

In contrast to *Daphnia*, heterotrophic protists grow efficiently on bacterial diets (Sherr & Sherr, 1984; Fenchel, 1986), which suggests that they do not require a dietary source of essential lipids. Both heterotrophic nanoflagellates and ciliates have been shown to upgrade cyanobacterial carbon for subsequent use by *Daphnia*, which has been attributed to the *de novo* synthesis of essential lipids by the protozoans (Martin Creuzburg *et al.*, 2005a, 2006; Bec *et al.*, 2006) and/or to the detoxification of cyanobacterial food (Bec *et al.*, 2006). This adds to the general view that heterotrophic protists play a significant role in transferring prokaryotic (picocyanobacterial and bacterial) carbon to higher trophic levels (Sherr & Sherr, 1984). Metazoan grazers differ significantly in their ability to feed on bacteria. In contrast to copepods, which prey inefficiently on small particles ($< 5\,\mu m$), daphnids nonselectively feed on a large size range of particles, which includes bacteria and bacterivorous protozoans (DeMott, 1986; Jürgens, 1994). Hence, copepods may indirectly use (already upgraded) bacterial carbon by consuming bacterivorous protozoans, whereas *Daphnia* species directly feed on the nutritionally inadequate bacteria, which may have consequences for the zooplankton community structure, in particular in meso to oligotrophic systems with a high share of bacterial carbon. However, even within the genus *Daphnia*, species differ in their efficiency to filter feed on natural bacterioplankton (Gophen & Geller, 1984) and, considering our data, this suggests that species able to efficiently exploit bacterial food are particularly prone to a limitation by sterols. On the other hand, besides the deficiency in essential lipids, bacteria may provide a significant source of carbon and elemental nutrients (Hessen & Andersen, 1990; Vadstein, 2000), so that a high share of bacterial carbon within the edible size fraction of Lake Seston may reduce carbon or phosphorus limitation and hence may increase the population growth of *Daphnia*, especially in oligotrophic lakes (Hessen & Andersen, 1990). It remains to be tested how different mixtures of algae and bacteria support somatic and population growth of *Daphnia* species under various environmental conditions (e.g. nutrient availabilities). Moreover, the diversity of heterotrophic bacteria in freshwater ecosystems is high and even strains belonging to the same species often differ considerably in their ecophysiology, for example, in their capability to produce potentially toxic secondary metabolites (Lengeler *et al.*, 1999). Hence, more studies are required before we are able to assess the significance of heterotrophic bacteria for the nutrition of *Daphnia* in the field.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389 3402.

Atlas RM (1997) *Handbook of Microbiological Media*. CRC Press, Boca Raton, FL.

Azam F, Fenchel T, Field JG, Gray JS, Meyer Reil LA & Thingstad F (1983) The ecological role of water column microbes in the sea. *Mar Ecol Prog Ser* **10**: 257 263.

Bec A, Martin Creuzburg D & Von Elert E (2006) Trophic upgrading of autotrophic picoplankton by the heterotrophic nanoflagellate *Paraphysomonas* sp. *Limnol Oceanogr* **51**: 1699 1707.

Brendelberger H (1991) Filter mesh size of cladocerans predicts retention efficiency for bacteria. *Limnol Oceanogr* **36**: 884 894.

Degans H, Zöllner E, Van der Gucht K, De Meester L & Jürgens K (2002) Rapid *Daphnia* mediated changes in microbial community structure: an experimental study. *FEMS Microbiol Ecol* **42**: 137 149.

Deines P, Matz C & Jürgens K (2009) Toxicity of violacein producing bacteria fed to bacterivorous freshwater plankton. *Limnol Oceanogr* **54**: 1343 1352.

DeMott WR (1986) The role of taste in food selection by freshwater zooplankton. *Oecologia* **69**: 334 340.

von Elert E (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnol Oceanogr* **47**: 1764 1773.

Fenchel T (1986) The ecology of heterotrophic microflagellates. *Adv Microb Ecol* **9**: 57 97.

Freese HM, Eggert A, Garland JL & Schumann R (2010) Substrate utilization profiles of bacterial strains in plankton from the River Warnow, a humic and eutrophic river in North Germany. *Microb Ecol* **59**: 59 75.

Glöckner FO, Zaichikov E, Belkova N, Denissova L, Pernthaler J, Pernthaler A & Amann R (2000) Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. *Appl Environ Microb* **66**: 5053 5065.

Gophen M & Geller W (1984) Filter mesh size and food particle uptake by *Daphnia*. *Oecologia* **64**: 408 412.

Grieneisen ML (1994) Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. *Insect Biochem Molec* **24**: 115 132.

Hall TA (1999) Bioedit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* **41**: 95 98.

Harrison KE (1990) The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. *J Shellfish Res* **9**: 1 28.

Hessen DO (1985) The relation between bacterial carbon and dissolved humic compounds in oligotrophic lakes. *FEMS Microbiol Lett* **31**: 215 223.

Hessen DO & Andersen T (1990) Bacteria as a source of phosphorus for zooplankton. *Hydrobiologia* **206**: 217 223.

Jander G, Rahme LG & Ausubel FM (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J Bacteriol* **182**: 3843 3845.

Jensen PR & Fenical W (1994) Strategies for the discovery of secondary metabolites from marine bacteria ecological perspectives. *Annu Rev Microbiol* **48**: 559 584.

Jürgens K (1994) Impact of *Daphnia* on planktonic microbial food webs a review. *Mar Microb Food Webs* **8**: 295 324.

Jürgens K, Arndt H & Rothhaupt KO (1994) Zooplankton mediated changes of bacterial community structure. *Microb Ecol* **27**: 27 42.

Lampert W (1991) The dynamics of *Daphnia* in a shallow lake. *Verh Int Ver Limnol* **24**: 795 798.

Langenheder S & Jürgens K (2001) Regulation of bacterial biomass and community structure by metazoan and protozoan predation. *Limnol Oceanogr* **46**: 121 134.

Lapteva NA, Bel'kova NL & Parfenova VV (2007) Spatial distribution and species composition of prosthecate bacteria in Lake Baikal. *Microbiology* **76**: 480 486.

Lengeler JW, Drews G & Schlegel HG (1999) *Biology of the Prokaryotes*. Thieme, Stuttgart.

Martin Creuzburg D, Bec A & Von Elert E (2005a) Trophic upgrading of picocyanobacterial carbon by ciliates for nutrition of *Daphnia magna*. *Aquat Microb Ecol* **41**: 271 280.

Martin Creuzburg D, Wacker A & Von Elert E (2005b) Life history consequences of sterol availability in the aquatic keystone species *Daphnia*. *Oecologia* **144**: 362 372.

Martin Creuzburg D, Bec A & Von Elert E (2006) Supplementation with sterols improves food quality of a ciliate for *Daphnia magna*. *Protist* **157**: 477 486.

Martin Creuzburg D, Westerlund SA & Hoffmann KH (2007) Ecdysteroid levels in *Daphnia magna* during a molt cycle: determination by radioimmunoassay (RIA) and liquid chromatography mass spectrometry (LC MS). *Gen Comp Endocr* **151**: 66 71.

Martin Creuzburg D, Von Elert E & Hoffmann KH (2008) Nutritional constraints at the cyanobacteria *Daphnia magna* interface: the role of sterols. *Limnol Oceanogr* **53**: 456 468.

Martin Creuzburg D, Sperfeld E & Wacker A (2009) Colimitation of a freshwater herbivore by sterols and polyunsaturated fatty acids. *P R Soc B* **276**: 1805 1814.

Martin Creuzburg D, Wacker A & Basen T (2010) Interactions between limiting nutrients: consequences for somatic and population growth of *Daphnia magna*. *Limnol Oceanogr* **55**: 2597 2607.

Matz C & Kjelleberg S (2005) Off the hook how bacteria survive protozoan grazing. *Trends Microbiol* **13**: 302 307.

Matz C, Webb JS, Schupp PJ, Phang SY, Penesyan A, Egan S, Steinberg P & Kjelleberg S (2008) Marine biofilm bacteria evade eukaryotic predation by targeted chemical defence. *PLoS One* **3**: e2744.

Napolitano GE (1998) Fatty acids as trophic and chemical markers. *Lipids in Freshwater Ecosystems* (Arts MT & Wainman BC, eds), pp. 21 44. Springer, New York.

Pace ML & Cole JJ (1994) Comparative and experimental approaches to top down and bottom up regulation of bacteria. *Microb Ecol* **28**: 181 193.

Padmanabhan V, Prabakaran G, Paily KP & Balaraman K (2005) Toxicity of a mosquitocidal metabolite of *Pseudomonas fluorescens* on larvae & pupae of the house fly, *Musca domestica*. *Indian J Med Res* **121**: 116 119.

Pearce DA, van der Gast CJ, Woodward K & Newsham KK (2005) Significant changes in the bacterioplankton community structure of a maritime Antarctic freshwater lake following nutrient enrichment. *Microbiology* **151**: 3237 3248.

Perga ME, Kainz M, Matthews B & Mazumder A (2006) Carbon pathways to zooplankton: insights from the combined use of stable isotope and fatty acid biomarkers. *Freshwater Biol* **51**: 2041 2051.

Qi W, Nong G, Preston JF, Ben Ami F & Ebert D (2009) Comparative metagenomics of *Daphnia* symbionts. *BMC Genomics* **10**: 172.

Russell NJ & Nichols DS (1999) Polyunsaturated fatty acids in marine bacteria a dogma rewritten. *Microbiology* **145**: 767 779.

Sherr BF & Sherr EB (1984) Role of heterotrophic protozoa in carbon and energy flow in aquatic ecosystems. *Current Perspectives in Microbial Ecology* (Klug MJ & Reddy CA, eds), pp. 412 423. American Society for Microbiology, Washington, DC.

Simon M, Cho BC & Azam F (1992) Significance of bacterial biomass in lakes and the ocean: comparison to phytoplankton biomass and biogeochemical implications. *Mar Ecol Prog Ser* **86**: 103 110.

Sinden SL, DeVay JE & Backman PA (1971) Properties of syringomycin, a wide spectrum antibiotic and phytotoxin produced by *Pseudomonas syringae*, and its role in the bacterial canker disease of peach trees. *Physiol Plant Pathol* **1**: 199 213.

Sterner RW (1997) Modelling interactions of food quality and quantity in homeostatic consumers. *Freshwater Biol* **38**: 473 482.

Summons RE, Bradley AS, Jahnke LL & Waldbauer JR (2006) Steroids, triterpenoids and molecular oxygen. *Philos T R Soc B* **361**: 951 968.

Taipale S, Kankaala P, Tiirola M & Jones RI (2008) Whole lake dissolved inorganic ^{13}C additions reveal seasonal shifts in zooplankton diet. *Ecology* **89**: 463 474.

Taipale S, Kankaala P, Hämäläinen H & Jones RI (2009) Seasonal shifts in the diet of lake zooplankton revealed by phospholipid fatty acid analysis. *Freshwater Biol* **54**: 90 104.

Tan MW, Mahajan Miklos S & Ausubel FM (1999) Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *P Natl Acad Sci USA* **96**: 715 720.

Vadstein O (2000) Heterotrophic, planktonic bacteria and cycling of phosphorus phosphorus requirements, competitive ability, and food web interactions. *Advances in Microbial Ecology* (Schink B, ed), pp. 115 167. Kluwer Academic/ Plenum Publishers, New York.

Van der Gucht K, Vandekerckhove T, Vloemans N, Cousin S, Muylaert K, Sabbe K, Gillis M, Declerk S, De Meester L & Vyverman W (2005) Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiol Ecol* **53**: 205 220.

Vodovar N, Vinals M, Liehl P, Basset A, Degrouard J, Spellman P, Boccard F & Lemaitre B (2005) *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species. *P Natl Acad Sci USA* **102**: 11414 11419.

Volkman JK (2003) Sterols in microorganisms. *Appl Microbiol Biot* **60**: 495 506.

Wacker A & von Elert E (2001) Polyunsaturated fatty acids: evidence for non substitutable biochemical resources in *Daphnia galeata*. *Ecology* **82**: 2507 2520.

Widdel F & Pfennig N (1981) Studies on dissimilatory sulfate reducing bacteria that decompose fatty acids. I. Isolation of new sulfate reducing bacteria with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. *Arch Microbiol* **129**: 395 400.

Ziprin R & Hartman PA (1971) Toxicity of *Pseudomonas aeruginosa* bacterins and cell walls to the greater wax moth, *Galleria mellonella*. *J Invertebr Pathol* **17**: 265 269.